International Journal of Biological Macromolecules 107 (2018) 730–740

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effects of annealing on the physical properties of therapeutic proteins during freeze drying process

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ARTICLE INFO

Article history:
Received 23 August 2017
Received in revised form 9 September 2017
Accepted 10 September 2017
Available online 20 September 2017

Keywords:
Freeze drying
Annealing
Etanercept
Mannitol
Glycine
Biophysical properties

ABSTRACT

Effects of annealing steps during the freeze drying process on etanercept, model protein, were evaluated using various analytical methods. The annealing was introduced in three different ways depending on time and temperature. Residual water contents of dried cakes varied from 2.91% to 6.39% and decreased when the annealing step was adopted, suggesting that they are directly affected by the freeze drying methods Moreover, the samples were more homogenous when annealing was adopted. Transition temperatures of the excipients (sucrose, mannitol, and glycine) were dependent on the freeze drying steps. Size exclusion chromatography showed that monomer contents were high when annealing was adopted and also they decreased less after thermal storage at 60 °C. Dynamic light scattering results exhibited that annealing can be helpful in inhibiting aggregation and that thermal storage of freeze-dried samples preferably induced fragmentation over aggregation. Shift of circular dichroism spectrum and of the contents of etanercept secondary structure was observed with different freeze drying steps and thermal storage conditions. All analytical results suggest that the physicochemical properties of etanercept formulation can differ in response to different freeze drying steps and that annealing is beneficial for maintaining stability of protein and reducing the time of freeze drying process.

1. Introduction

Even though the development of biotechnologically engineered therapeutic proteins has significantly increased, development stages, particularly those related to protein formulation, still face challenges including protein aggregation, physicochemical instability, and undesirable pharmacokinetic properties like short half-life. In addition, stresses affecting the physicochemical stability of proteins may occur during various processes, such as manufacturing, purification, shipping, and storage [1,2]. The stability of therapeutic proteins is dependent on various internal factors, such as buffer with specific pH, protein concentration, and excipients as exposed to temperature, agitation, and oxidation [3–11]. For these reasons, obtaining enough protein stability has been considered as one of the priorities during the development. To achieve long-term stability, a solid-state dosage form after freeze drying has been preferred. A lot of commercially available products have already adopted the process to prepare solid-state protein products [12–14].

The freeze drying process mainly comprises freezing, primary drying, and secondary drying. The freezing step is important as it establishes the morphology of the frozen protein formulation; this may affect the duration of next drying steps [15,16]. With respect to the processing time, the optimization of freeze drying cycle is mostly focused on reducing the primary drying time, the longest step of the freeze drying process [17]. During primary drying, proper shelf temperature and chamber pressure need to be established for removing ice from the frozen formulation by sublimation, resulting in a structurally intact dried cake [18]. With those essential processes, the annealing step can be adopted optionally after early freezing to fully crystalize widely used excipients.

The annealing process is a part of the freezing step wherein the frozen formulation is kept above the final freezing temperature for a defined time [19,20]. The annealing temperature needs to be between the melting temperature of ice and Tg', and holding times can vary depending on the formulation and heating rate [14,20,21]. Actually, the annealing step is optional according to the formulation; however, it has been widely adopted in the process to stabilize physicochemical properties of protein formulations. Annealing can be beneficial for the formulation, including that of some amorphous materials, such as mannitol and glycine, which may form a metastable glass and do not entirely crystallize during the initial freezing step. If these materials are not sufficiently crystallized,

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https://doi.org/10.1016/j.ijbiomac.2017.09.041
0141-8130/

they can subsequently crystallize during storage and release the associated moisture, and this moisture can destabilize the protein.

Annealing also affects the particle size distribution of the ice crystal. The size of ice crystals smaller than a critical size decrease and accordingly larger ones are formed as ruled by Ostwald ripening [17,20]. Therefore, the increased crystal size of ice and the decreased mass transfer resistance by the dried layer can accelerate the sublimation rate, with enlarged pore diameter preoccupied by the ice crystals [22]. Another effect of annealing is that heterogeneous sublimation rates that occur between samples during the freezing step can be reduced by implementing the annealing step; this results in homogeneous cake structures between vials and increases the primary drying efficiency [23,24]. Therefore, annealing can be a valuable step and may aid complete crystallization of the materials.

The annealing step has widely been incorporated as a part of the freeze drying process of protein formulations. Removing amorphous phase in a crystalline component increases the Tg', thus improving the primary drying efficiency at high temperatures [14,25]. Annealing in glycine:sucrose (1:1, w/w) formulation showed increase in Tg' from −44 °C to −33 °C, making a more solid formulation [26,27]. Annealing induced a more native-like secondary structure in rhIFN-γ formulation and improved its appearance by eliminating cracks in dried cakes [28]. It also suppressed the air–liquid interface formation related with rhIFN-γ aggregation on reconstitution. Annealing was also applied to decrease the duration of primary drying by ~38% in insulin-loaded nanoparticles; it also decreased the overall freeze drying time by ~26% [17].

Despite the advantages of the annealing step in the freeze drying process, its effect on protein formulation cannot be easily predicted [29]. In addition, the influence of annealing on the structural stability of proteins still has yet to be studied in detail [17]. Annealing did not noticeably alter the secondary structure and storage stability of IgG1 formulations, including sucrose [30]. Although it is generally accepted that an annealing temperature above Tg' can cause protein unfolding, this effect was negligible in freeze-concentrated systems [31]. Therefore, the benefit of the annealing step needs careful assessment according to the formulation.

Herein, different freeze drying steps with and without annealing were applied to evaluate its effects on protein stability. For this evaluation, various analytical methods were used, including Karl Fisher titration for water content, scanning electron microscopy (SEM) for morphology, differential scanning calorimetry (DSC) for thermodynamic factors of materials, circular dichroism (CD) for protein secondary structure, dynamic light scattering (DLS) for the Z-average size, and size exclusion chromatography (SEC) for content changes including fragments, monomers, and aggregates of protein.

2. Materials and methods

2.1. Materials

Etanercept, the model drug used in this study, is a dimeric fusion protein consisting of 934 amino acids and is composed of human 75 tumor necrosis factor receptor (TNFR) as an extracellular ligand-binding portion connected with the Fc region of human IgG1 including the C_H2, C_H3, and hinge regions. Enbrel® is a commercial name of etanercept and was kindly donated by the Seoul National University (Seoul, South Korea). L-Histidine, D-mannitol, sucrose, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, sodium chloride, hydranal®-water standard 10.0, and hydranal®-composite 5 were purchased from Sigma–Aldrich (St. Louis, MO, USA). L-Histidine hydrochloride monohydrate was obtained from the Junsei Chemical (Tokyo, Japan). Glycine was obtained from Duchefa Biochemie (Haarlem, Netherlands). All other reagents were of analytical grade and used as received.

2.2. Sample preparation

The model drug was dissolved in 1 mL water for injection (WFI) and then dialyzed three times in 20 mM pH 6.6 histidine buffer for 24 h at 4 °C in a CelluSep® F2 cellulose membrane, having a MW cut-off of 6000–8000 Da (Membrane Filtration Products, Seguin, TX, USA). The histidine buffer included 1% sucrose and 4% mannitol or glycine as excipients. For aseptic preparation of samples, protein samples were prepared under a laminar flow cabinet (Biofree, Seoul, South Korea). The protein concentration in each solution was adjusted to 5.0 mg/mL.

2.3. Freeze drying processes

Etanercept samples were transferred into semi-stoppered glass vials and freeze-dried in a FDUT-6002 compact type lyophilizer from Operon (Gyeonggi, South Korea). The samples were frozen by ramp cooling from 20 °C to −40 °C for 2 h and then maintained at −40 °C for another 2 h. Drying conditions were as follows: primary drying was done at −25 °C for 24 h, followed by secondary drying at 20 °C for 8 h (without annealing (WA) sample, totally 36 h for freeze drying) (Table 1).

The thermal treatment of annealing was introduced in the freeze drying process in three different ways. First, as introduced in the WA sample, samples were frozen from 20 °C to −40 °C for 2 h, and the temperature was increased to the annealing temperature of −15 °C and held for 3 h. Then, the samples were cooled down to −40 °C and held for another 2 h. Primary drying was done at −25 °C for 16 h, followed by secondary drying (−25 °C primary drying, PD-1). Secondly, samples were frozen down to −40 °C for 2 h and the

Table 1
Various conditions of freeze-drying process for the study.

Conditions of freeze-drying process					
Process conditions	Freezing	Annealing	Primary drying	Secondary drying	Total time
Without annealing (WA)	Ramp cooling from 20 to −40 °C for 2 h and keep for another 2 h	–[a]	−25 °C for 24 h	20 °C for 8 h	36 h
−25 °C primary drying (PD-1)	Ramp cooling from 20 to −40 °C for 2 h	−15 °C for 3 h followed by maintaining at −40 °C for 2h	−25 °C for 16 h		31 h
−18 °C primary drying (PD-2)		−15 °C for 3 h followed by maintaining at −40 °C for 2h	−18 °C for 16 h		
−13 °C primary drying (PD-3)		−10 °C for 3 h followed by maintaining at −40 °C for 2h	−13 °C for 16 h		

[a] Annealing was not adopted in this method.